INTRODUCTION: Bisphosphonates are widely used clinically to treat osteoporosis and localize preferentially to bone (1). Although alendronate and risedronate, two FDA-approved bisphosphonates, have been shown to be effective in preventing bone resorption in in vitro and in vivo models, their exact mechanism of action remains unknown. The effects of bisphosphonates on osteoclasts have been studied, but there is limited information on the effects of bisphosphonates on osteoblast proliferation and activity. Recently, Mundy et al. demonstrated that statins, inhibitors of HMG CoA reductase in the mevalonate pathway, enhance BMP-2 gene expression and bone formation in a mouse calvaria model (2). Since bisphosphonates also inhibit different reactions in the mevalonate pathway, we hypothesized that bisphosphonates may have an anabolic effect on osteoblasts. Our canine model established that in addition to bone loss, bisphosphonates enhanced bone ingrowth into implants as well. Thus, the purpose of this study was to find direct in vitro evidence on whether alendronate and risedronate have anabolic effects on osteoblasts.

MATERIALS AND METHODS: We used MG-63 human osteoblast-like cells co-cultured in α-MEM media (Biowhittaker) supplemented with 10% FBS, 100U/ml penicillin, 100µg/ml streptomycin, 25µg/ml amphotericin B, and L-glutamine (2 mM) at 37°C with 95% humidity and 5% CO2. Alendronate (Merck) and risedronate (Procter & Gamble) were added at varying concentrations to different cultures and changed at 7 and 14 days.

1) Assessment of cell proliferation: A MTT colorimetric assay augmented direct cell counting as a means to assess cell proliferation. For the MTT assay, cells were plated at a density of 2.5 x 10^4 cells/well in 96-well plates. Alendronate and risedronate were added in varying concentrations from [10^-10] to [10^-7]M. The MTT assay was completed in triplicate for each sample and repeated in six cultures. Cells were also plated at a density of 2.5 x 10^5 cells/well in 12-well plates, bisphosphonates were added to the culture media in concentrations ranging from [10^-9] to [10^-6]M, and cell proliferation was assessed by direct cell counting. Direct cell counts were conducted in duplicate for each sample and repeated in five cultures.

2) Alkaline phosphatase assay: Effects of bisphosphonates on osteoblast maturation were investigated with an alkaline phosphatase bioassay after 24, 48, and 72 hours after treatment. The production of alkaline phosphatase synthesis was measured by the conversion of a colorless p-nitrophenyl phosphate to a colored p-nitrophenol (Sigma). All experiments were conducted in triplicate and repeated in 5 cultures.

3) RT-PCR: Total RNA was extracted using the TRIzol® reagent (GibcoBRL) at the 3rd, 5th, 7th, 10th, 14th, and 21st day after treatment. Reverse transcription-polymerase chain reaction (RT-PCR) using PCR beads (Amersham Pharmacia Biotech) helped determine the presence of gene activity for the following markers: type I collagen, osteocalcin, BMP-2, BMP-4, and BMP-7 (Sigma).

4) Von Kossa staining: Mineralization of the matrix was studied using Von Kossa’s staining (Sigma). Cultures were performed at a density of 2.5 x 10^5 cells per well in a 12-well plate. Alendronate, risedronate, and vitamin D were added at concentrations of [10^-5]M. On the 14th and 21st days of the experiment the cells were fixed with 1M sodium cacodylate and then washed. Silver nitrate (5%) was added to the cell cultures in a dark room followed by exposure to UV light for 30 minutes. The data on cell proliferation and alkaline phosphatase was analyzed by a one-way ANOVA and a Student’s two t-test. All p-values were compared to a t-value of .05 to determine significance. RT-PCR and Von Kossa staining were compared qualitatively.

RESULTS: Both of the bisphosphonates evaluated, alendronate and risedronate, increased cell proliferation as measured by the MTT assay at the concentrations [10^-10]M to [10^-7]M (Fig.1). Direct cell counting for alendronate and risedronate treated media indicated a peak of cell proliferation at a concentration of [10^-5]M (30% and 38%, respectively, (p<.05). The bisphosphonates also increased alkaline phosphatase levels after 24, 48, and 72 hours of culture to comparable levels (p<.05). Further, after the administration of alendronate and risedronate RT-PCR confirmed active gene expression of type I collagen, osteocalcin, BMP-2, BMP-4, and BMP-7 mRNAs (Fig. 2). Von Kossa staining provided evidence of mineralization, but the technique was not sensitive enough to determine any differences due to the bisphosphonate treatment.

DISCUSSION: Our studies demonstrated that bisphosphonates have a discernable anabolic effect on osteoblast-like cells. MG-63 cells showed an increase in osteoblast proliferation with increasing bisphosphonates concentrations, and a peak effect at [10^-5]M that is supported by the work of Giuliani et al (3). The expression of several genes related to bone formation indicates that in the presence of bisphosphonates overall osteoblast proliferation and maturation continues. Although further investigations are necessary to identify the specific mechanisms, animal models are crucial to confirm that bisphosphonates enhance osteoblastic activity in vivo. These results may lead to the use of bisphosphonates for enhancing bone ingrowth into implants, as well as preventing peri-implant bone resorption (4,5).

Acknowledgement: We thank J Kneeny and S Qureshi for their contributions.

Reference: